Effect of Early Weight-Bearing Training on Blood-Spinal Cord Barrier Function in Mice

Honors Research Distinction Thesis

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Abstract

Spinal cord injury (SCI) results in a breakdown of the blood-spinal cord barrier (BSCB) that permits a robust inflammatory response responsible for further damage to neural tissue. Neurotoxicity is thought to result from movement of inflammatory cells into the spinal cord through the damaged and permeable blood vessels. Activities such as treadmill training attempt to utilize spinal plasticity to promote recovery, but recent animal studies have shown increased BSCB permeability with early swim training. Exercise-regulated matrix metalloproteinase-9 (MMP-9) is a potent regulator of vascular permeability known to increase acutely following SCI, degrading endothelial tight-junctions. Bloodborne leukocytes may then extravasate into spinal cord tissue. Therefore, the goal of this study was to elucidate whether acute weight-bearing exercise contributes to greater BSCB permeability after SCI through MMP-9 activity. Vascular permeability after moderate/severe (75 kilodynes) contusive SCI at T9 was evaluated in both C57BL/6 wild type (WT) and MMP-9 null (KO) mice. Groups include treadmill-trained and untrained WT and untrained KO mice that survived 1 or 7 days post-injury (dpi). BSCB permeability at injury epicenter was identified using vascular delivery of Evans Blue Dye (EBD) and quantified via fluorescent stereology. Functional recovery was determined using the Basso Mouse Scale for Locomotion (BMS). We demonstrated markedly lower permeability at the lesion with treadmill training than without at 7 dpi in WT mice. In KO mice, we saw much less permeability, as expected. Similar differences remote to the epicenter were not observed. However, functional recovery was much lower following training at 7 dpi. Our results help establish potential cellular effects of activity-based interventions after SCI. Such findings may lead to the development of a more effective timecourse of treatment for human SCI patients to maximize functional recovery.

Introduction

Each year an estimated 12,000 people in the United States sustain a permanently disabling spinal cord injury (SCI) [1]. SCI results in immediate damage to both the neural tissue as well as the infiltrating vasculature near the site of injury. There has been a great deal of evidence showing that spinal cord injuries are not solely the result of the initial injury, but also a complex sequence of secondary events that occur acutely post-injury, and often remotely from the site of initial trauma [2]. These secondary events can be broadly characterized as the hemorrhaging of blood into the spinal column at the site of injury and an increased permeability of the intact vasculature of the spinal cord [3]. As a result of the blood-spinal cord barrier (BSCB) dysfunction present in the surrounding vasculature, inflammatory cells and molecules can gain access to the neurons of the spinal cord, causing further damage to neuronal tissue [4,5,6,7].

The time course of the increased BSCB permeability has been shown to peak at 3 days post-injury in rats, referred to as the acute post-injury phase [2]. Acutely initiated swim training in rats has been demonstrated to result in a further increase in permeability of the BSCB, and in a separate trial resulted in significantly poorer recovery of weight-bearing locomotion, a primary functional outcome measure [8]. It has been hypothesized by Smith, et al., that the lack of weight supported recovery is due to an increase in secondary pathogenesis due to the increased permeability brought on by training [8]. Physical activities such as treadmill training are routine in the treatment of humans, attempting to utilize the plasticity of the spinal cord to promote recovery [9]. While the previous studies show increased acute, dysfunction of the BSCB resulting from swimming (weight-supported) activity, they do not address the effects of weight-

bearing training on macromolecular extravasation into the spinal cord immediately following trauma. Additionally, the impact of barrier permeability on functional recovery of weight-bearing locomotion following task specific training and the effect of exercise on activity of enzymes known to be active following injury, particularly matrix metalloproteinases (MMPs), remains unknown.

The activity of MMPs and the role that they play in the breakdown of the BSCB after central nervous system injury has been well documented [4,5,6,7,10]. MMPs are known to be potent contributors to increasing the permeability of capillaries through the cleavage of occludins that hold endothelial cells together. MMP activity is known to be greatly heightened acutely after SCI, especially at the epicenter of the injury. Previous work by Guo, et al., has shown that exercise prior to central nervous system injury decreased the activity of MMP-9 and increases the presence and activity of tissue inhibitors of metalloproteinases (TIMPs) [11]. It is not known, however, if exercise following SCI exacerbates the increase of MMPs, leading to a larger degree of macromolecular extravasation into the spinal cord, and thus a greater inflammatory response.

This study proposes to examine the effect that acute, weight-bearing treadmill training has on the macromolecular permeability of the BSCB after a moderate-severe contusive SCI in mice. It also aims to relate the observed permeability with the resulting functional outcomes.

Materials and Methods

All procedures were performed under protocol approved by The Ohio State University Animal Care and Use Committee.

Subjects

C57BL/6 (WT) and MMP-9 null (KO) mice were included in this study. The WT mice were randomly assigned to three groups: 1 day post-injury (dpi) survival receiving no exercise (UX) (n=3), 7 dpi survival UX (n=4), and 7 dpi survival with exercise (EX) (n=5). The KO mice were placed into one group: 7 dpi survival UX (n=3). Group sizes were determined via power analysis. The mice were housed 4 per cage, given access to food and water ad libitum, and exposed to a daily 12 hour light/dark cycle.

Pre-Injury Training and Testing

Both WT and KO mice were acclimated to the treadmill training apparatus (Figure 1) prior to injury through subjection to three sessions totaling 25 minutes. Each animal was administered the Basso Mouse Scale for Locomotion (BMS) test one day prior to injury [12]. Mice that demonstrated cooperative behavior on the treadmill, and a BMS score of 9 (out of 9) indicating normal gait with no preexisting deficits, were deemed fit for inclusion in the study.

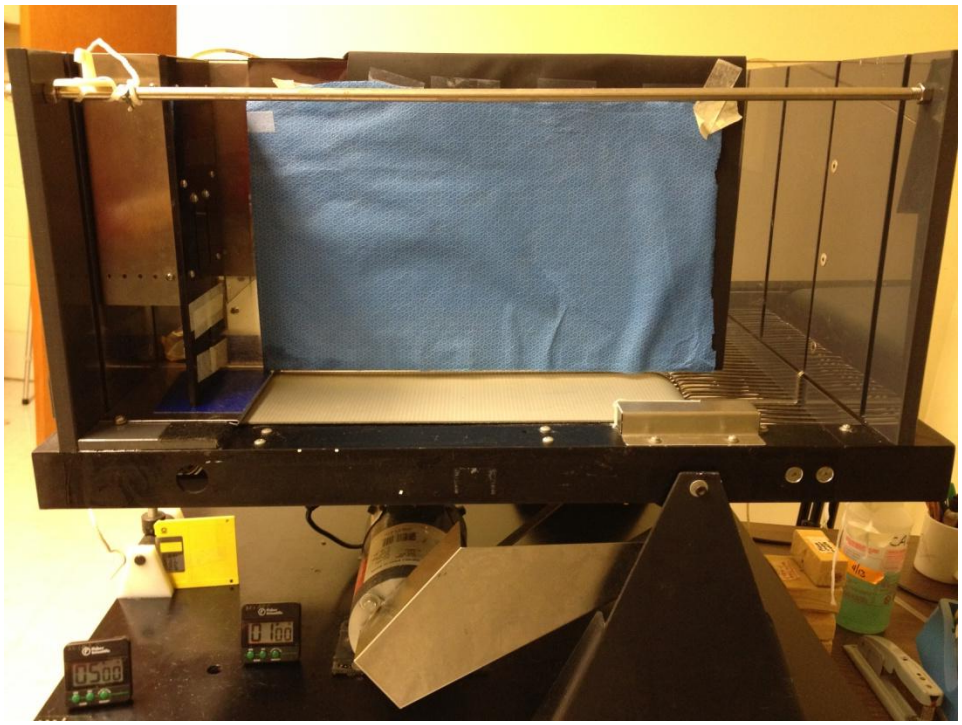

Figure 1. The treadmill training apparatus. Visible are the walls enclosing the belt, the electric motor that drives the belt, and the belt itself (grey).

Spinal Cord Injury

Mice receiving injuries were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg) administered intraperitoneally, followed by subcutaneous injection of prophylactic antibiotic gentomycin (1 mg/kg) and saline (2 cc). A dorsal laminectony was performed at the T9 spinal level, exposing the spinal cord (Figure 2A). The spinal column was stabilized via spinous process clamps at both T8 and T10. A moderate-severe, bilateral contusion injury was delivered to the dorsal aspect of the spinal cord with the Infinite Horizon (IH) Impactor (Precision Systems and Instrumentation, LLC, Fairfax Station, VA) with a programmed force of 75 kilodynes (Figure 2B). The wound was then sutured in layers and the animals placed in a heated recovery cage for 24 hours, as described by Smith, et al. **[**8].

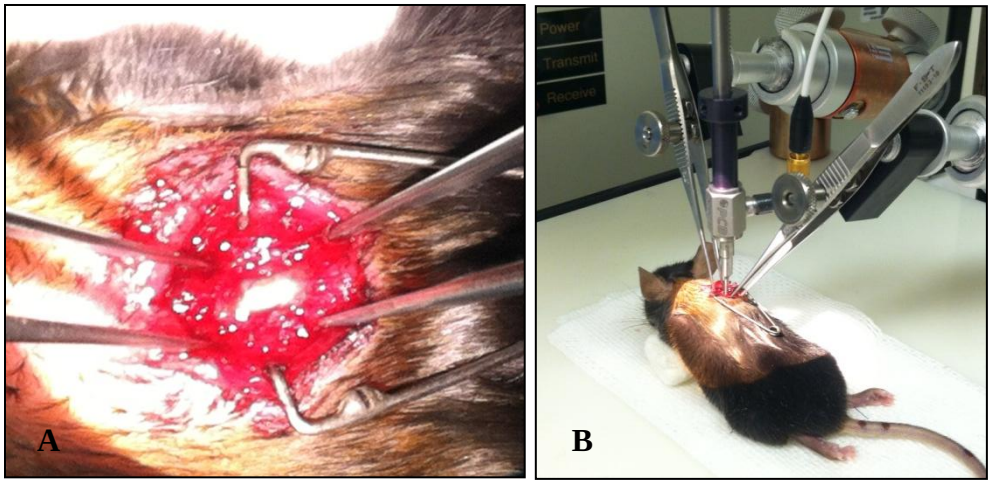

Figure 2. **A.** Exposed spinal cord tissue after incision along dorsal midline and removal of the dorsal lamina at T9. Note the stabilizing spinous process clamps attached at the T8 and T10 vertebrae. **B.** Anesthetized mouse stabilized beneath the impactor probe of the Infinity Horizon device, prepared to receive the injury.

Training Paradigm

One, two, and seven days post-injury (if in 7 dpi survival group), each animal was tested with the BMS to establish a post-injury baseline for determination of successful injury administration and assessment of functional recovery. Mice with scores greater than 1.5 at one day post-injury were eliminated from the study due to concern with injury uniformity. Mice assigned to receive treadmill training received twice daily training sessions of 10 minutes each, separated by 20 minutes, beginning two days post-injury and concluding at six days post-injury. The treadmill training was assisted through use of a minimally weight-supporting harness and physical cuing of the hind legs into a proper gait pattern.

Dye Administration and Tissue Collection

Evans Blue Dye (EBD) was selected as the measure of BSCB permeability due to its high binding affinity to blood borne albumin and large size that does not allow the dye to permeate

the intact BSCB. At the end of their assigned survival time (one or seven days), the mice were anesthetized (see *Spinal Cord Injury*) and injected either intravenously (IV) via the lateral tail vein or intraperitoneally (IP) with EBD in normal saline (2%, 4 mL/kg), as described by Manaenko, et al. [13]. The dye was allowed to circulate for 30 minutes while the animal remained under anesthetic [13]. The mice were then sacrificed via phosphate-buffered saline (0.1 M) cardiac perfusion, followed by paraformaldehyde (4%) fixative. The dorsal laminae of the spine were removed, and the entirety of the spinal cord from five millimeters rostral to five millimeters caudal of the epicenter collected. All samples were cryoprotected in sucrose (30%), blocked in M-1 mounting media (Thermo Scientific, Waltham, MA), and transversely sectioned at a thickness of 20 micrometers.

White Matter Sparing

Sectioned tissue was stained for white matter sparing (WMS) with eriochrome cyanin (EC) under the same procedure previously demonstrated in multiple studies [8,14]. The tissue was analyzed under light microscopy and converted to a computerized image (MCID-Elite, Imaging Research, Ontario). The proportion of spared white matter was calculated by outlining the entirety of the tissue section, as well as the spared white matter within the parenchyma. White matter was considered spared if the deep blue EC stain on the myelin was dense and continuous. The tissue section with the smallest proportion of spared white matter with relation to the area of white matter in naïve samples at the level injuries were delivered was determined to be the lesion site (epicenter).

Confocal Microscopy

The presence of EBD was detected via confocal microscopy (Olympus FluoView FV1000) at a fluorescent excitation wavelength of 633 nm. This excitation wavelength was chosen because it was the closest available setting on the confocal microscope to the 620 nm absorption peak shown by Saria, et al. [15].

Proportional Analysis of EBD Permeability

The relative area of EBD penetration into the parenchyma of the spinal cord was calculated at the previously determined epicenter, as well as sites 2.4 mm distant in both the rostral and caudal directions. The relative area was calculated using ImageJ (freely available at http://rsb.info.nih.gov/ij/, courtesy of the National Institute of Health) analyses of images obtained via confocal microscopy.

Statistical Analysis

The BMS, WMS, and EBD penetration data were subjected to a one-way ANOVA analysis between experimental groups. Post-hoc analysis for the differences between groups was carried out with Tukey's test. All descriptive statistics are reported as means ± standard error of the mean. Significance was determined at $p < .05$.

Results

The BMS data for all 7 day survival groups was analyzed at one and seven days post-injury, with the one day group excluded due to lack of comparison of functional recovery. At the one day time point following surgery, all groups averaged a BMS score of one or less, meaning that they demonstrated only slight movement at the ankle. There were no statistical differences in the one day data. At the seven day time point, the average BMS scores demonstrated large differences in functional recovery between the three groups (Figure 3).

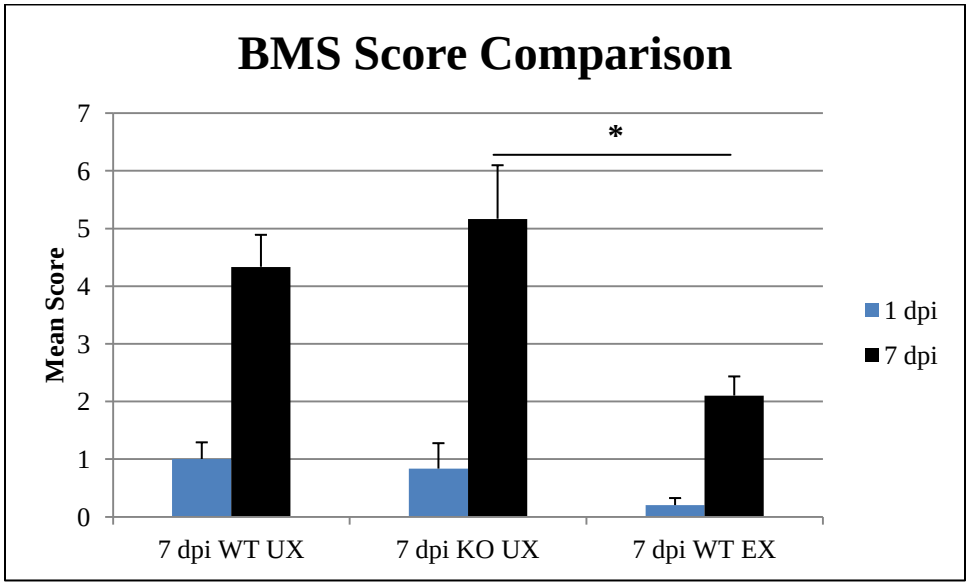

Figure 3. Comparison of BMS mean scores by group at both one and seven days post-injury. *Indicates significance.

The 7 dpi KO UX group showed the greatest degree of functional recovery, followed closely by the 7 dpi WT UX group. The performance of the 7 dpi WT EX group at seven days was significantly poorer than that of the KO group ($p<.05$), averaging three points lower. When compared to the WT UX animals, the EX group scored over two points worse, though the difference was not statistically significant ($p= .073$). At the seven day time point, the KO group showed consistent plantar stepping with some coordination on average. The 7 dpi WT UX

animals presented with occasional plantar stepping and no coordination of gait, while the 7 dpi WT EX mice demonstrated only extensive movement at the ankle with no plantar stepping or coordination of gait.

Analysis of the four experimental groups showed the greatest degree of WMS at the lesion in the 1 dpi WT group and the least in the 7 dpi WT EX mice when normalized against the mean area of white matter naïve samples near T9 (Figure 4). There was little difference between the 7 dpi groups, but there was an evidently greater degree of sparing in the 7 dpi KO UX group. However, none of the groups showed any statistical difference.

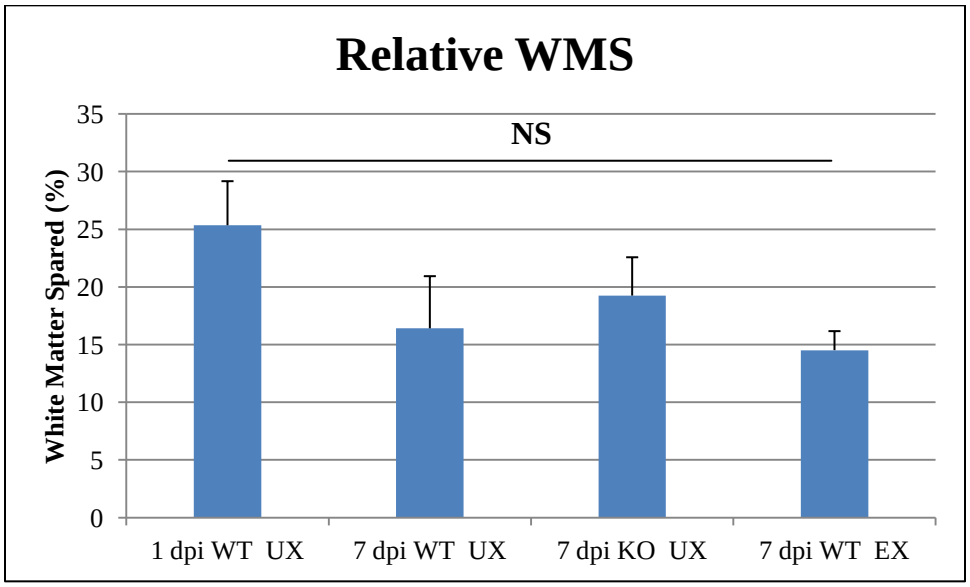

Figure 4. Comparison of spared white matter at the epicenter, relative to the average area of white matter in naïve animals near T9. Not statistical (NS).

The Evans Blue imaging clearly showed the presence of the dye in the spinal cord tissue of all experimental groups, both at the epicenter and distant to the lesion. There appeared to be a close correlation between the greatest presence of EBD and the section identified as the lesion site by

the WMS analysis. EBD was present in the spinal cord of some subjects up to 4.8 millimeters distant from the epicenter, though to varying degrees. Qualitatively, The 7 dpi KO UX group showed the least EBD both at the level of and distant to the epicenter, while the 1 dpi WT UX group showed the greatest presence in both locations, in some cases completely covering the tissue section. Naïve samples showed no permeability of the spinal cord, as was expected based on previous work by Whetstone, et al. [16]. Images for representative animals showing the EBD fluorescence signal and anatomy at varying levels of the spinal cord can be found in Appendix A. The level of the lesion can be identified by the presence of a third image of the spinal cord section stained with EC that was used in the WMS analysis.

Quantitative analysis of the tissue for the presence of EBD verified the qualitative assessments of the EBD penetration. Proportional analysis at the epicenter demonstrated large differences in EBD penetration between groups (Figure 5). Both the 1 dpi WT UX and 7 dpi WT UX groups showed significantly greater proportional areas of EBD than the 7 dpi KO UX animals at the epicenter ($p < .05$). The 7 dpi WT EX animals presented values much closer to the 7 dpi KO UX, showing no significant difference between the two. The 7 dpi WT EX did not show statistical difference to either the 1 dpi or 7 dpi WT UX ($p = .081$ and $p = .074$, respectively), but did show much less permeability.

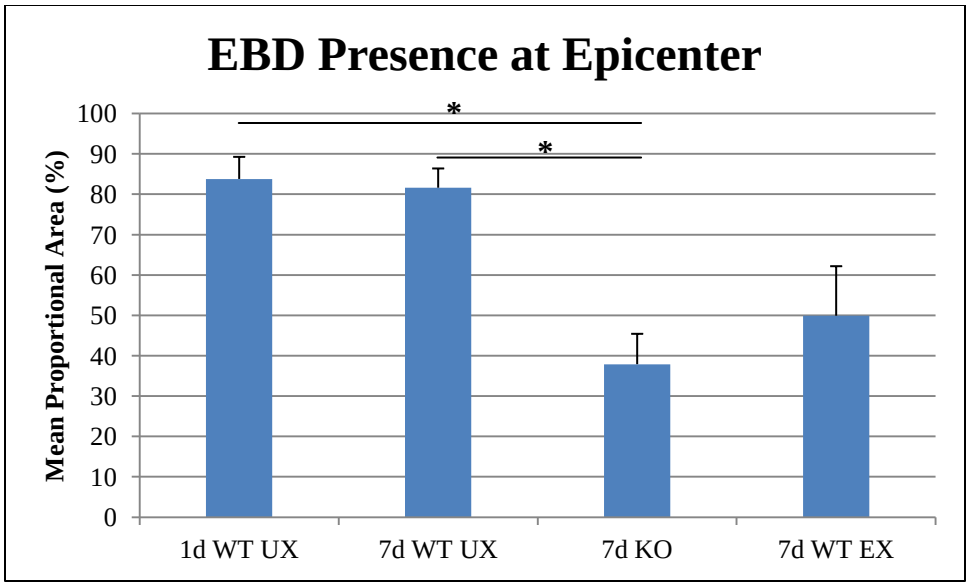

Figure 5: Mean proportional area of EBD presence at the epicenter for all groups. *Indicates significance.

Analysis of the tissue sections 2.4 mm rostral to the epicenter showed a large decrease in proportional area of EBD across all experimental groups, ranging from 25 to 60% (Figure 6). The largest decreases were seen in the 7 dpi groups. The 7dpi KO UX group presented with the lowest proportional area, remaining slightly below the presence detected for the 7 dpi WT UX and 7 dpi WT EX. The 1 dpi WT UX group showed significantly greater EBD in the parenchyma than all other groups ($p < .05$). No other statistically significant differences were identified.

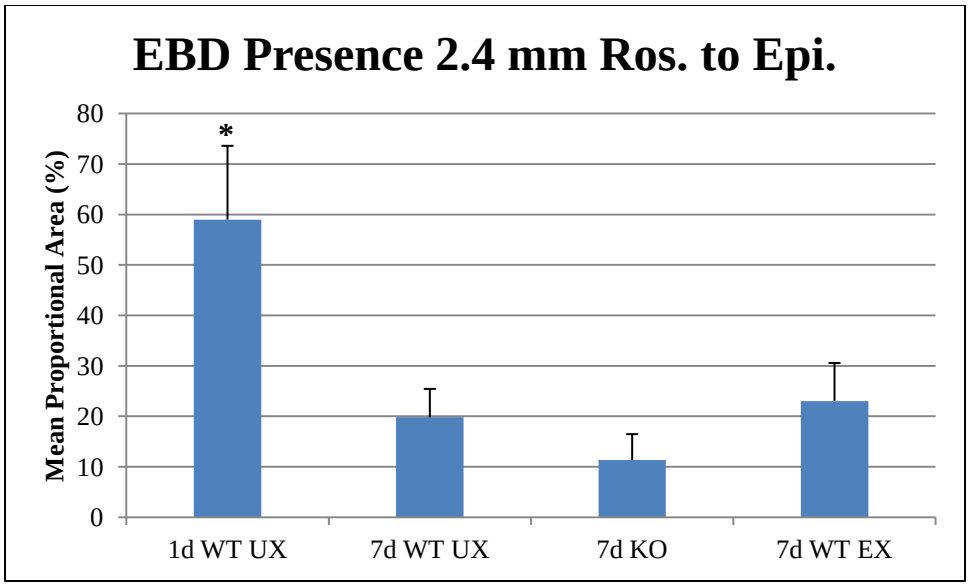

Figure 6: Mean proportional area of EBD presence 2.4 mm rostral to the lesion site across all experimental groups. *Indicates significance.

Analysis of the images 2.4 mm caudal to the epicenter showed a similar trend to the rostral data, both in the large decrease of EBD presence across all groups and the relative amount of EBD presented by each group (Figure 7). The 1 dpi WT UX animals again demonstrated the greatest degree of permeability, significantly higher than all other experimental groups ($p < .05$). While not statistically significant to the remaining 7 dpi groups, the 7 dpi KO UX mice showed the least permeability and presence of EBD of all groups.

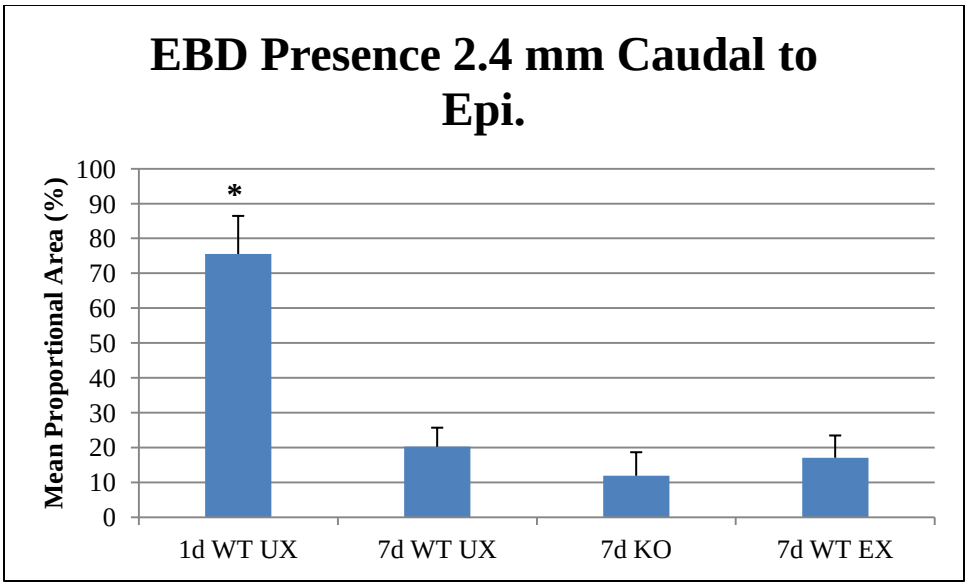

Figure 7: Mean proportional area of EBD presence 2.4 mm caudal to the lesion site across all experimental groups. *Indicates significance.

Discussion

The analysis of EBD area in the sectional images at the epicenter, 2.4 mm rostral, and 2.4 mm caudal to the epicenter showed greater permeability for every group in comparison with naïve samples. The 1 dpi WT UX mice showed the greatest permeability at all locations by a large margin, both rostrally and caudally. This difference can be attributed to the traumatic nature of the injury itself. A moderate/severe injury causes massive physical trauma to a large region of the cord beyond the epicenter, severing blood vessels and creating a great deal of primary hemorrhage not due to cellular changes in permeability. This hemorrhage is responsible for the overwhelming presence of EBD in the parenchyma of the spinal cord. In comparison, 7 dpi groups were given sufficient time for the hemorrhaging to cease at locations distant to the epicenter and the material to be removed from the parenchyma.

Between the 7 dpi groups, there was little difference in the proportional area of EBD at the rostral and caudal locations with respect to the lesion. This was supported by the work of Popovich, et al., who showed that the maximum permeability of the spinal cord at these locations is slow developing and not reached until approximately day 14 [2]. This suggested that the overall picture of permeability at these locations was not given enough time to elucidate any differences between groups. Smith, et al., found similar results distant to the epicenter, showing that the differences between groups were not statistically significant [8]. Worth noting, however, was the permeability of the KO group, which presented the lowest of the groups. This was supported by work that has shown MMP-9 to be one of the primary modulators of the BSCB and permeability [4,5,6,7,10].

The epicenter of the injury across all 7 dpi groups showed permeability for the WT UX group that was significantly higher than that of the KO group and nearly the WT EX animals. The KO and WT EX animals showed no statistical difference in mean. This difference between the two WT groups was counterintuitive to the results of Smith, et al., who demonstrated that the initiation of acute swim training following SCI resulted in drastically increased permeability at the epicenter [8]. This makes sense when realizing that an increase in physical exertion would lead to an increase in heart rate and blood pressure. An increase in both of these parameters could feasibly create greater permeability. There are several possible explanations for the very different results that arose from the two studies. It may be that swimming is a much more physically demanding task than terrestrial locomotion. Swimming involves greater stability of the trunk and areas near the epicenter of injury than treadmill training with a harness. Increased muscle activation in these areas may increase blood demand and vasodilation that carries over

into the spinal circulation nearby. Also, swimming is not as natural as terrestrial locomotion, as rats and mice are terrestrial rodents. A stress response could have resulted in the swimming rats that contributed further to heart rate and blood pressure increases, creating the greater permeability.

The similarity seen between the KO and WT EX permeability at the lesion could indicate a connection between exercise and MMP-9 attenuation. Research by Arima, et al., has showed that sensory pathways originating in the lower limb in response to weight-bearing have an effect on permeability of the BSC, particularly at the L5 level of the spinal cord [17]. At this level of the cord, the lumbar enlargement (the primary connection with muscles of the lower limb), they demonstrated that there was a measurable change in the permeability of various inflammatory cells in response to stimuli, suggesting that MMP-9 may have been at least partly responsible. Guo, et al., presented that there was a correlation between pre-injury terrestrial exercise and decrease in permeability due to MMP-9 attenuation by tissue inhibitors of metalloproteinases (TIMPs) [11]. Assuming this effect holds true for post-injury training, this mediation of MMP-9 levels may only be present as a result of weight-bearing training, as was conducted by Arima, et al. [17]. The sensory pathways may interact with the TIMPs present at the site of injury, reducing permeability. In this case, this result would not be seen for swim trained animals.

WMS demonstrated no statistical difference between any of the groups, similar to the data reported by Smith, et al. [8]. Though not significant, the spared white matter was greatest in the 1 dpi WT UX group. This may possibly be explained by the progression of the neural death after

SCI. Initially, only the neurons damaged by the physical trauma are killed. However, with the abundance of severed blood vessels at the site of injury, it has been shown that ischemia in the remaining tissue no longer receiving blood is a major contributor to secondary neural death [4]. Seven days is sufficient time for this process to have killed a much larger number of cells than at one day, resulting in less spared white matter in the 7 dpi animals. An argument could also be made for the actions of inflammatory cells at the lesion site leading to less spared white matter at 7 dpi versus 1 dpi. As mentioned previously, inflammatory cells such as macrophages and neutrophils have been shown to utilize the disrupted BSCB to gain access to the parenchyma of the spinal cord [4,5,6,7,10]. Detloff, et al., also showed that as a result of these infiltrates, resident microglia are often activated at and below the level of injury [18]. The inflammatory mechanisms affected by these cells cause further cell death through oxidative breakdown of cellular components. The progression of these mechanisms through 7 dpi would lead to a greater breakdown in the white matter than the relatively brief exposure that would be expected at 1 dpi.

While we demonstrated a large decrease in permeability at the epicenter in the exercised group against the unexercised at 7 dpi, the functional recovery, as scored by the BMS, was less complete in the exercised group. This appears counterintuitive, as the greater degree of permeability in the unexercised group, as previously evidenced, would lead to a greater inflammatory response and neural damage. Smith, et al., showed a similar effect in their swimming trials in which trained rats exhibiting greater BSCB dysfunction at the lesion site scored more poorly in open-field locomotor tests [8]. It was evident that there must have been another mechanism at work in the weight-bearing, treadmill-trained mice that affected the spinal cord independently of epicenter permeability that was not present in the swimming model

(weight-supported). A possible explanation may lie in the study by Arima, et al., in which they demonstrated that permeability at the lumbar enlargement was increased by the stimulus of weight-bearing on the hind limbs [17]. This evidence points to the possibility that in a weight-bearing training model, permeability and inflammatory response at the lumbar enlargement may have a larger impact on the recovery of functional locomotion than the same effects at the epicenter in the thoracic region.

The finding that permeability was decreased at the epicenter with acutely initiated, weight-bearing exercise, yet the functional recovery of overground locomotion was decreased could present serious implications with regard to current human therapeutic interventions. There are many rehabilitation centers, including the NeuroRecovery Network with which I am affiliated here at The Ohio State University, that utilize treadmill-based locomotor therapy similar to that described by this study in the treatment of cervical and thoracic spinal cord injuries. This primary motivation of this intervention is to regain functional abilities, primarily walking. Assuming that training begun too acutely could have similar effects in human patients as shown in our results, premature intervention may prove harmful to the overarching goal of functional recovery after SCI. It appears that this form of training could be a novel approach to attenuating vascular permeability at the epicenter, possibly sparing a greater proportion of neural tissue due to lessened inflammation. However, more work must be conducted to better elucidate the connections between lumbar enlargement permeability and possible decreased functional recovery, alongside similar work already being conducted at the level of the lesion. Of particular importance is the acute timeframe at which functional recovery is reduced with training, so that

interventions may be conducted at a time appropriate to affect both lessened permeability at the epicenter and functional recovery of gait.

Conclusions

From this study, we were able to determine that weight-bearing exercise administered acutely post-SCI resulted in less permeability of the spinal cord vasculature at the epicenter, but made no difference in cord sections distant to the epicenter, when compared to the 7 dpi WT UX group. We were also able to confirm that the lack of MMP-9 (in the KO mice) results in significantly less vascular permeability. The exercise effect can be postulated to be correlated with the activity of MMP-9 due to the similar presentation of the 7 dpi KO and WT EX. One possibility is the upregulation of tissue inhibitors of metalloproteinases (TIMPs) that has been shown with similar exercise. Though permeability decreased in trained animals, this groups BMS functional outcome scores were poorer than both the 7 dpi WT UX and KO groups. The significant disparity between the WT EX animals and the other groups points to a mechanism of regaining functional ability that is separate from cell processes responsible for lessening permeability. Together, exercise and its possible regulation of MMP-9 may be a novel approach to attenuate vascular permeation events acutely following SCI that can result in further neural damage. However, this approach and its benefits appear to necessitate a balance with the possibility of more limited functional recovery as a result.

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Appendix A

Representative Tissue Sections for Each Group

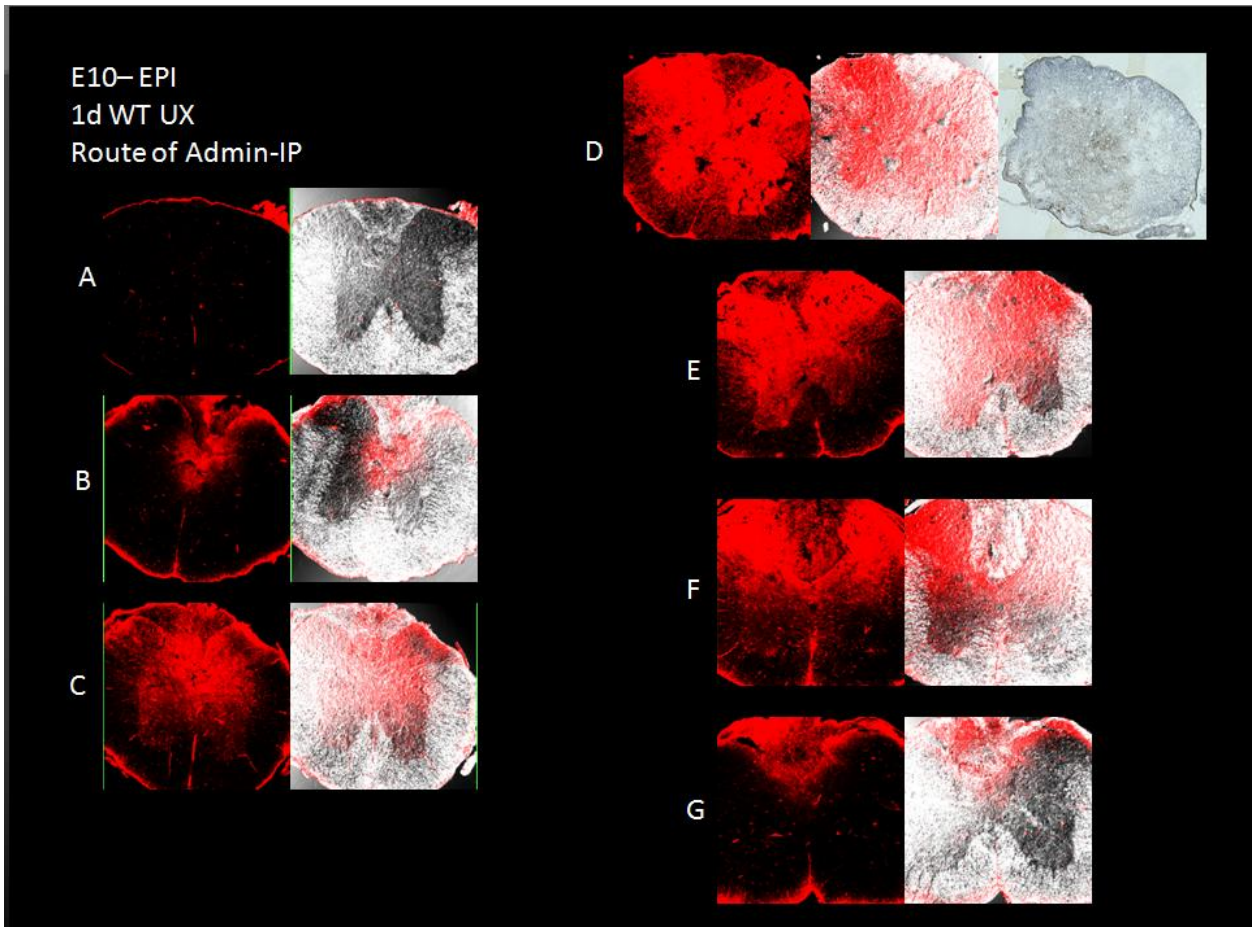

Figure A1. Tissue sections from animal number E10, representative of individuals from the 1 dpi WT UX group. Sections A-G represent sections oriented rostrally-caudally. Epicenter is present at section D. First image shows EBD fluorescence (red), second image shows grey and white matter anatomy, and third image (at epicenter) shows the lesion (areas without blue EC stain). Note the widespread presence of EBD both at and distal to the epicenter.

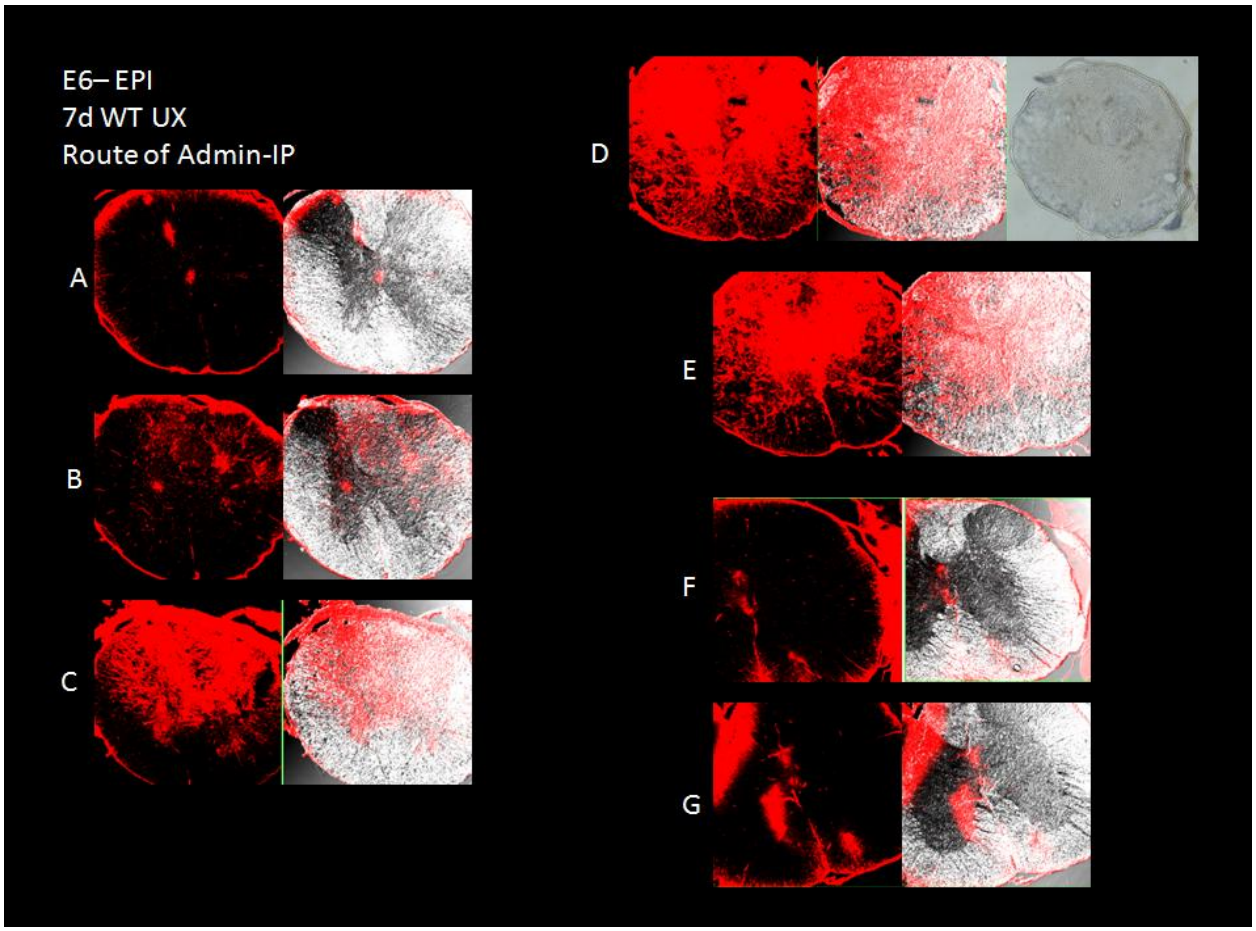

Figure A2. Tissue sections from animal number E6, representative of individuals from the 7 dpi WT UX group. Sections A-G represent sections oriented rostrally-caudally. Epicenter is present at section D. First image shows EBD fluorescence (red), second image shows grey and white matter anatomy, and third image (at epicenter) shows the lesion (areas without blue EC stain).

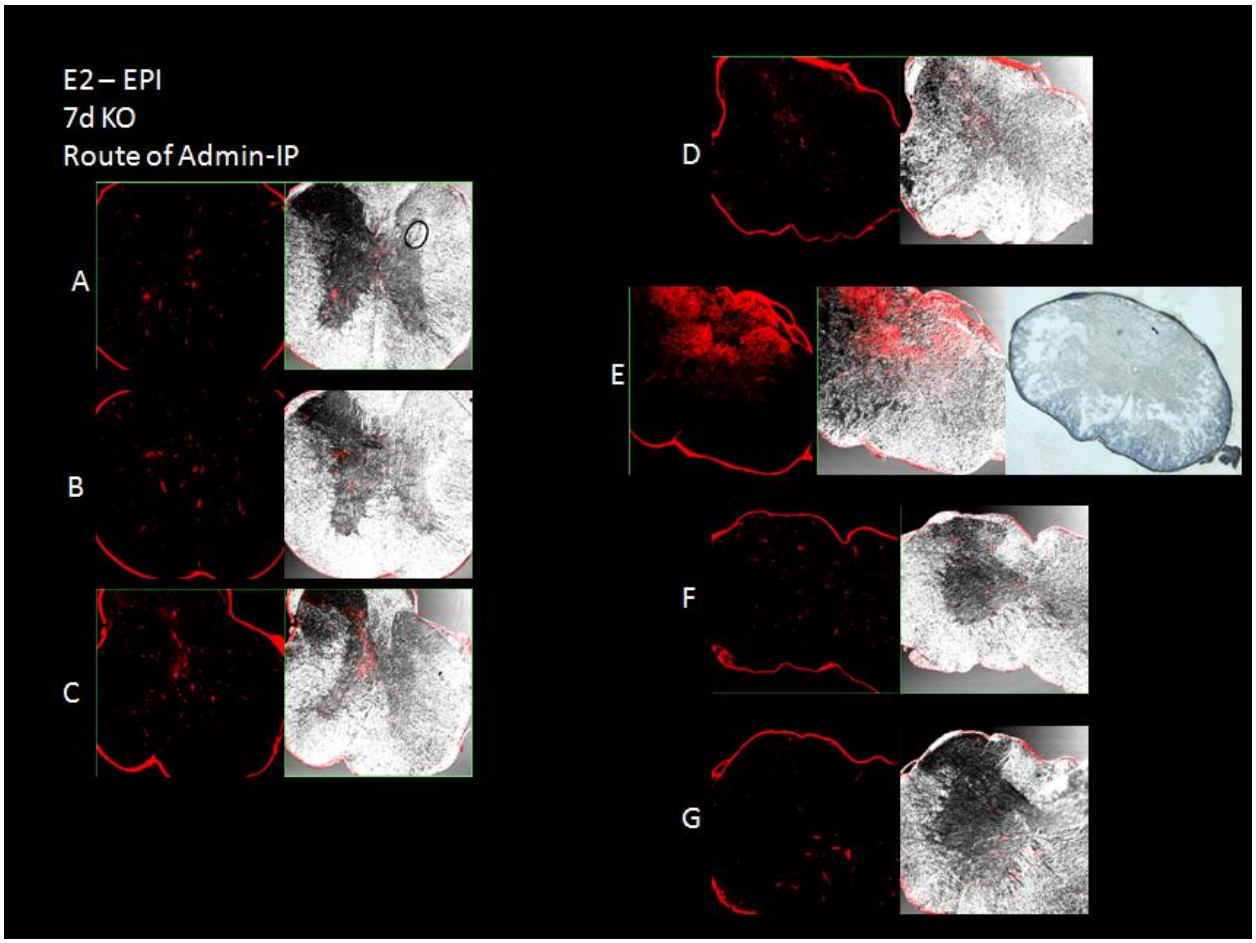

Figure A3: Tissue sections from animal number E2, representative of individuals from the 7 dpi KO UX group. Sections A-G represent sections oriented rostrally-caudally. Epicenter is present at section E. First image shows EBD fluorescence (red), second image shows grey and white matter anatomy, and third image (at epicenter) shows the lesion (areas without blue EC stain). Note the relative lack of EBD presence both at and distal to the epicenter.

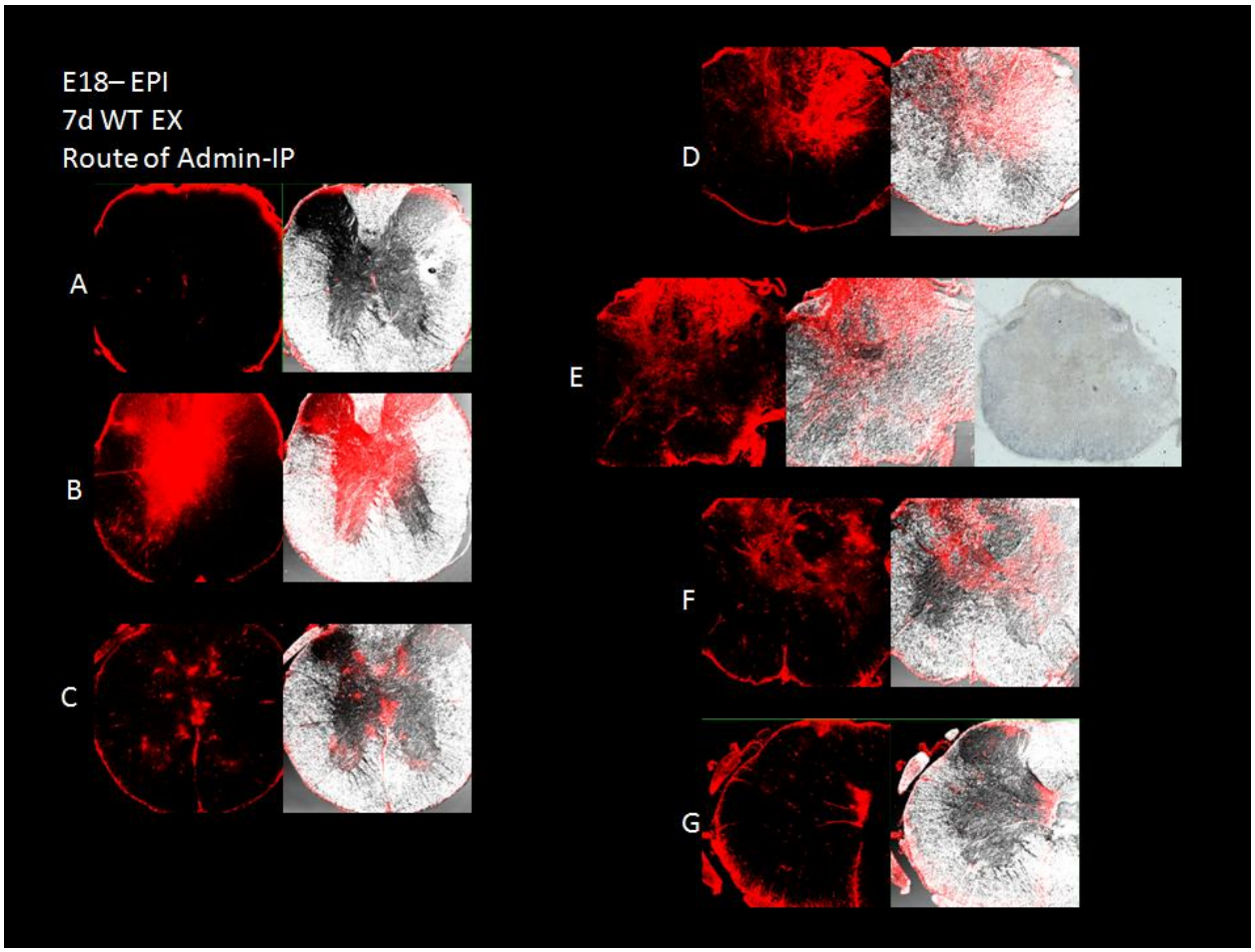

Figure A4: Tissue sections from animal number E18, representative of individuals from the 7 dpi WT EX group. Sections A-G represent sections oriented rostrally-caudally. Epicenter is present at section E. First image shows EBD fluorescence (red), second image shows grey and white matter anatomy, and third image (at epicenter) shows the lesion (areas without blue EC stain). Note the relative lack of EBD presence both at and distal to the epicenter when compared to the representative animal of the 7 dpi WT UX group (E6) in Figure A2.